Am. J. Hum. Genet. 43:414–421, 1988

Intermediate Homocysteinemia: A Thermolabile Variant of Methylenetetrahydrofolate Reductase

Soo-Sang Kang, Jeimin Zhou, Paul W. K. Wong, John Kowalisyn, and Gary Strokosch

Department of Pediatrics, Rush Medical College and Presbyterian-St. Luke's Medical Center, Chicago

Summary

A "newly detected" variant of methylenetetrahydrofolate (MTHF) reductase (E.C.1.1.1.68) deficiency associated with an 8–15-fold increase in plasma total homocysteine was discovered in two unrelated patients who had subnormal serum folate. However, the homocysteinemia was corrected by oral folic acid supplement. When MTHF reductase activities in lymphocyte extracts before and after heat treatment at 46 C for 5 min were compared, there was a consistent difference in heat stability between the enzyme from the controls and that from the patients. The mean residual activities after heat treatment were 37.0% (34.1%–42.6%) in the controls and 15.2% and 15.1% in the two patients, respectively. Two obligate heterozygotes for severe MTHF reductase deficiency had residual activities of 39.6% and 37.7%. A similar difference in thermostability was demonstrated in cultured skin fibroblasts and lymphoblasts. Studies with a mixture of lymphoblast extracts from a control and a patient and with partially purified enzyme suggested that the thermostability was an independent characteristic of MTHF reductase. These observations provided evidence of a hitherto undescribed mutant MTHF reductase in our two patients with intermediate homocysteinemia. Unlike previously reported patients with MTHF reductase deficiency, there was no apparent clinical problem related to the abnormal folate or homocysteine metabolism during infancy or childhood in these two subjects, but one of them had vascular disorders in adulthood. The observations in these two subjects suggested that a moderate deficiency of MTHF reductase might be associated with vascular disorders in adult life.

Introduction

Homocysteine is an unstable molecule that is readily oxidized to homocystine (homocysteine–homocysteine disulfide) and cysteine-homocysteine mixed disulfide in the plasma. In addition, these free disulfides are spontaneously converted to protein-bound forms. Since about 80% of plasma homocysteine is present in the protein-bound form in nonhomocystinuric subjects (Kang et al. 1979), the determination of free homocystine in the supernatant after acid precipitation of plasma detects only severe homocystinemia. However, a 30–60-fold increase of plasma in vivo protein-bound homocyst(e)ine has been demonstrated in homocystinuric patients. Hence it is plausible that a less severe degree of homocysteinemia may be found by the determination of protein-bound homocyst(e)ine.

Homocystinurias are autosomal recessive disorders resulting from a metabolic block in either the conversion of homocysteine to cystathionine or the remethylation of homocysteine to methionine (Mudd et al. 1972; Mudd and Levy 1983; Rosenberg 1983; Erbe 1986; Cooper and Rosenblatt 1987). These include deficiency of cystathionine synthase, which converts homocysteine to cystathionine; methylenetetrahydrofolate (MTHF) reductase, which converts MTHF to methyltetrahydrofolate; and defects in the metabolism of methylcobalamin, which is the coenzyme of methionine synthase in the remethylation of homocysteine (fig. 1).

Mutation of genes responsible for these enzymes may produce moderate enzyme deficiency, resulting in mild

Received July 23, 1987; revision received April 11, 1988.

Address for correspondence and reprints: Dr. Soo-Sang Kang, Section of Genetics, Department of Pediatrics, Rush Medical College, 1750 West Harrison Street, Chicago, IL 60612.

0002-9297/88/4304-0007$02.00

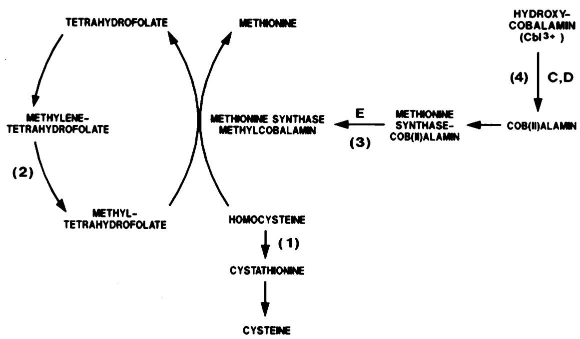

Figure 1 Metabolic pathways involved in homocysteinemia. Metabolic defects include cystathionine synthase deficiency (1), methylenetetrahydrofolate reductase deficiency (2) and mutations in cobalamin metabolism (3 and 4).

to moderate homocysteinemia, which can be detected by the determination of protein-bound homocyst(e)ine. On the other hand, nutritional deficiency of pyridoxine or folic acid also produces moderate homocysteinemia (Smolin and Benevenga 1982; Kang et al. 1987). It is also known that methionine loading increases the concentration of plasma homocystine and cysteine–homocysteine disulfide (Sardharwalla et al. 1974). Therefore, the combination of moderate deficiency of any of the above enzymes and inappropriate nutritional factors involved in homocysteine metabolism may increase the severity of homocysteinemia.

In the present paper, intermediate homocysteinemics are defined as subjects with a 3–20-fold increase of plasma total homocysteine. Of the two subjects with intermediate homocysteinemia reported in the present study, one was found among patients with coronary artery disease and the other was found among subjects with low serum folate. Biochemical studies in these subjects provided evidence for genetic heterogeneity of MTHF reductase mutations that resulted in intermediate homocysteinemia.

Material and Methods

Patients

Patient I, a white male school teacher, had a positive family history of coronary artery disease. His first attack of myocardial infarction occurred at 35 years of age, and two subsequent attacks occurred at 37 and 41 years of age. He also had a cerebrovascular accident at the age of 45 years. A mild coronary artery disease was demonstrated by angiography at age 36, and definite stenosis in three coronary arteries was found at the second (42 years of age) and third (46 years of age) investigations. At age 42, plasma total homocysteine was 35.9 nmol/ml (normal 4.25 $\pm$ 1.40) and plasma methionine was 27.0 nmol/ml (normal 12–30). Other laboratory data were as follows: serum folic acid, 1.4 ng/ml (normal 2–16); B12, 198 pg/ml (normal 190–1,000); total cholesterol, 212 mg/dl (normal 120–280); reptilase time, 14.5 s (normal 13–19); antithrombin III functional, 93% (normal 80–115; alpha-2-antiplasmin, 112% (normal 80–120); plasminogen, 97% (normal 75–120); protein C activity, 91% normal; fibronectin, normal; and mean corpuscular volume (MCV) of erythrocytes, 102 μm^3 (normal 82–92). No methylmalonic acid was detected in the urine. Cyanocobalamin uptake of cultured skin fibroblasts was 7.0 pg/10^6 cells, and its distribution was 4.6% as cyanocobalamin, 58.1% as methylcobalamin, and 10.7% as adenosylcobalamin, indicating no abnormality. In cultured skin fibroblast extracts, specific activity of cystathionine synthase was 10.7 nmol cystathionine formed/mg protein/h (controls' values 8.9, 9.3, and 14.6), specific activities of holoenzyme (without the addition of methylcobalamin) and total enzyme (with the addition of methylcobalamin) of methionine synthase were 4.4 nmol methionine formed/mg protein/h (controls' values 3.8, 4.6, and 4.8), and 7.6 nmol methionine formed/mg protein/h (controls' values 6.5, 7.1, and 8.5), respectively. This patient had been a cigarette smoker until 35 years of age but never had hypertension or diabetes. Daily multivitamin supplementation, including 1 mg of folic acid/day, was started at 45 years. His plasma total homocysteine was maintained within the normal range (4.7 nmol/ml at 45 years, 5.8 nmol/ml at 46 years). However, discontinuation of vitamin therapy for 12 wk caused a rebound of plasma total homocysteine to 12.7 nmol/ml. Family history revealed that his father had died of a heart attack at the age of 65 years. Of four siblings, a 42-year-old brother had myocardial infarction at 35 years of age and had a plasma total homocysteine level of 12.9 nmol/ml. His 72-year-old mother and a 44-year-old sister had normal plasma total homocysteine values. Further studies in the family members were refused.

Patient II, a black female, was hospitalized for viral pneumonia at 17 years of age. Complete blood count showed the following: red blood cells, 3.44 $\times$ 10^6/mm^3; hemoglobin, 9.9 g/dl; hematocrit, 30.9%; MCV, 90 μm^3; white blood cells, 17.2 $\times$ 10^3/mm^3 with 74% segmented neutrophile leukocytes. Serum cyanocobalamin and folate were 189 pg/ml and 1.1 ng/ml, respectively. Plasma total homocysteine was 61.1

nmol/ml (normal 4.25 ± 1.40), and plasma methionine was 30 nmol/ml (normal 12–30). Subsequently, all laboratory data except vitamin and homocysteine values returned to normal. Serum total cholesterol, uric acid, glucose, and pyridoxine levels were normal during and after the hospitalization. No methylmalonic acid was detected in the urine. Cyanocobalamin uptake of cultured skin fibroblasts was 5.8 pg/10^6 cells, and its distribution was 4.0% as cyanocobalamin, 62.1% as methylcobalamin, and 13.8% as adenosylcobalamin, indicating no abnormality. In cultured skin fibroblast extracts, specific activity of cystathionine synthase was 9.8 nmol cystathionine formed/mg protein/h (controls' values 8.9, 9.3, and 14.6), and specific activities of holoenzyme and total enzyme of methionine synthase were 4.6 nmol methionine formed/mg protein/h (controls' values 3.8, 4.6, 4.8) and 8.2 nmol methionine formed/mg protein/h (controls' values 6.5, 7.1, and 8.5), respectively. After two intramuscular injections of 1 mg cyanocobalamin with one weekly interval, serum B12 increased to 279 pg/ml and plasma total homocysteine decreased to 34.1 nmol/ml. After 4 wk of oral folic acid therapy with 1 mg/day, plasma total homocysteine was reduced to 6.5 nmol/ml. Family history revealed that her six siblings, from 12 to 27 years of age, and her parents, aged 52 and 53 years, had no clinical evidence of vascular disease. The family members refused all laboratory studies.

Serum Folic Acid, B12, and Protein-bound Homocysteine Analysis

The Becton Dickinson Simul TRAC®-S Radioassay kit was used for the quantitation of folic acid and B12 (Lou et al. 1965; Herbert et al. 1966; Dunn and Foster 1973; Rudzki et al. 1976). The concentration of total homocysteine was determined in serum samples stored for more than 4 wk at −22 C. The method of analysis was as described elsewhere (Kang et al. 1979, 1982). Total homocysteine was expressed as homocystine in nanomoles per milliliter of serum. To convert total homocysteine to homocystine, the value for total homocysteine was divided by two.

Cells

Lymphocytes, cultured lymphoblasts, and skin fibroblasts were used for the preparation of enzyme extracts. Ficoll-Hypaque was used for the isolation of lymphocytes from heparinized blood (Jondal et al. 1972). Epstein-Barr virus–transformed lymphoblast cell lines from patient I and a control subject were established by Dr. Bernard Strauss, Department of Microbiology, University of Chicago. Cell line GM 5854, obtained from the National Institutes of Health Genetic Mutant Repository in Camden, NJ, was also used as control. Lymphoblasts were cultured in RPMI 1640 containing 15% FCS (GIBCO) and were harvested in the log phase. Skin fibroblast cell lines were established from skin biopsies from the two patients and four controls. Fibroblasts were cultured until confluence in minimal essential medium containing 10% FCS and 1% glutamine. Harvesting was carried out with Hank's buffered solution by scraping with a rubber policeman. The cells were washed three times with Hank's buffer. After centrifugation at 10,000 *g* for 10 min, the cell pellets were stored at −75 C until the preparation of enzyme extracts.

Enzyme Assay

Stored cells were resuspended in 0.5–1.0 ml of 50-mM potassium phosphate buffer, pH 6.3, lysed by freezing and thawing three times, and centrifuged at 20,000 *g* for 30 min. The supernatant was used for enzyme analysis after dialysis against 500 vol 50 mM phosphate buffer, pH 7.2, overnight. In some experiments, the crude extracts were partially purified with ammonium sulfate precipitation (45% saturation) followed by adsorption to DEAE at pH 7.2 (Kutzbuch and Stockstad 1971). The ammonium sulfate precipitates were separated by centrifugation for 30 min at 15,000 *g* and dissolved in 50 mM phosphate buffer for dialysis against the same buffer overnight. The dialysate was then passed through DEAE, which had been previously equilibrated with 50 mM phosphate buffer, pH 7.2, washed with the same buffer three times, and then eluted with 0.3 M phosphate buffer, pH 7.2. The eluent from DEAE was dialyzed as described for the crude extracts. Protein concentration was determined by the method of Lowry et al. (1951).

MTHF reductase (methyltetrahydrofolate-menadione oxidoreductase) was assayed according to a modification of the methods described by Kutzbuch and Stockstad (1971) and Wong et al. (1977*a*). To remove radioactive impurities from commercially available [5–^{14}C] methyltetrahydrofolate, thin-layer chromatography with cellulose acetate plate, 50 mM ammonium bicarbonate, pH 8.0, and 0.1 M 2-mercaptoethanol was used. The purified substrate was stored in 34 nM ascorbate, pH 6.5. Without purification, the radioactive substrate produced high blank values, sometimes as high as 100,000 cpm in some batches. When the purified substrate was used, blank values were consistently less than 500 cpm. Another modification was the addition of 10 μl of 1.3 M formaldehyde at the end of incubation.

The reaction mixture consisted of 0.18 M potassium phosphate buffer, pH 6.3, 37 nM menadione bisulfite, 80 μM [5–^{14}C] methyltetrahydrofolate (0.8–1.4 × 10^6 cpm), 1.15 mM EDTA, pH 7.0, 11.5 mM ascorbic acid, plus or minus 54 μM flavin adenine dinucleotide (FAD) and 258 μl enzyme extract in a final volume of 436 μl. The amount of enzyme preparation in each tube was either 100 μg of the supernatant protein from the cell lysate or 20 μg of partially purified enzyme protein. Heat inactivation was performed by incubating the enzymes in a 46 C water bath for 5 min in 50 mM potassium phosphate buffer, pH 7.2. The reaction mixture with unheated or heated enzyme extracts was incubated at 37 C for 60 min. Then the reaction was terminated by the addition of 0.3 ml of 20 mM dimedone in 1.0 M potassium acetate buffer, pH 4.5, and 10 μl of 1.3 M formaldehyde at 0 C. The mixture was placed in boiling water for 5 min and then cooled in ice for 5 min. After the addition of 3 ml toluene, it was vigorously mixed with a Vortex mixer. After low-speed centrifugation, a 2-ml aliquot of the toluene phase was removed for the measurement of radioactivity. Enzyme activity was expressed in nanomoles of formaldehyde produced per milligram of protein per hour.

Results

In the absence of FAD the mean ± SD specific activity of lymphocyte MTHF reductase from nine normal subjects was 5.97 ± 1.77 nmol formaldehyde produced/mg protein/h. The activity increased by 24.3% in the presence of FAD (table 1). In contrast, the enzyme activities of lymphocyte extracts obtained from patients I and II were 35% and 38% of the normal mean, respectively, indicating a partial deficiency of MTHF reductase in these patients with intermediate homocysteinemia. Partial enzyme deficiency was detected in the absence or presence of FAD. In addition, heat-inactivation studies repeatedly showed a decreased thermostability in MTHF reductase from the two homocysteinemic subjects. When enzyme extracts were heated at 46 C for 5 min, the mean activity decreased to about 37.0% of the original in the nine control lymphocyte extracts and to about 15% of the original in the two patient lymphocyte extracts (table 1). The thermostability of MTHF reductase from obligate heterozygotes for severe MTHF reductase deficiency was not distinguishable from that of the controls (table 1).

A similar study was performed with cultured skin fibroblasts (table 2). Although MTHF reductase activities were highly variable before heat treatment, a similar thermostability was consistently observed in fibro-

Table 1

MTHF Reductase Activity in Lymphocyte Extracts Before and After Heat Treatment

Trial and Subject(s)	FAD in Assay Mixture	Enzyme Activity (nmol HCHO/mg protein/h)		Activity After Heat Treatment (%)
		Before Heat Treatment	After Heat Treatment	
I:				
Control[a]	−	5.97 ± 1.77 (4.07–8.12)	2.21 ± .74 (1.23–3.05)	37.0 (34.1–42.6)
Patient I	−	2.11	.32	15.2
Patient II	−	2.25	.34	15.1
Heterozygote I[b]	−	3.69	1.46	39.6
Heterozygote II[b]	−	3.13	1.18	37.7
II:				
Control[c]	−	7.63 (6.35–8.83)	...	...
	+	9.48 (8.55–10.66)	3.75 (3.41–4.20)	39.6 (35.0–42.4)
Patient I	−	2.98	...	...
	+	3.88	.64	16.5

Note.—Values in parenthesis indicate the range.
[a] Mean ± SD enzyme activity from nine normal subjects.
[b] Parents of patients with severe MTHF reductase deficiency.
[c] Means of values from three normal subjects.

Table 2

MTHF Reductase Activity in Cultured Skin Fibroblast Extracts Before and After Heat Treatment

Subject(s)	Enzyme Activity (nmol HCHO/mg protein/h) Before Heat Treatment	After Heat Treatment	Activity After Heat Treatment (%)
Control[a]	2.59 (1.30–3.43)	.98 (.52–1.30)	37.8 (36.5–40.0)
Patient I	3.28	.42	12.8
Patient II	1.83	.26	14.2

Note.—Values in parenthesis indicate the range.
[a] Mean of values obtained from four normal subjects.

blast extracts from both the controls and the patients, again demonstrting decreased thermostability in the enzyme from the patients.

There were also wide variations of MTHF reductase activities in cultured lymphoblast extracts from two control cell lines obtained from two different individuals (table 3). Furthermore, the enzyme activities of the patient's cell lines were higher than the activities of both controls' cell lines. However, heat-inactivation studies provided clear evidence of decreased thermostability in the MTHF reductase from the patient. The mixing experiment (V), using equal volumes of extracts from C225 and PO5, showed a residual activity of 24.1% after heat inactivation. The calculated residual activity was 24.0% (table 3). This observation suggested that thermostability was an independent characteristic of each enzyme.

MTHF reductase activity was also determined in partially purified fractions of lymphoblast extracts (table 4). The thermostability of the partially purified enzymes was similar to that of the crude extracts. This observation further strengthened the suggestion that thermostability was a property characteristic of the enzyme itself and was not due to any interaction with other components in the crude extracts.

Discussion

Severe MTHF reductase deficiency is characterized by neurological abnormalities, mental retardation, psychiatric symptoms, free homocystine, and relatively low methionine in the plasma and urine (Erbe 1979, 1986; Rowe 1983). A pathologic study in such a patient has demonstrated vascular thrombosis and changes simi-

Table 3

MTHF Reductase Activity in Cultured Lymphoblast Extracts Before and After Heat Treatment

Experiment, Strains	Enzyme Activity (nmol HCHO/mg protein/h) Before Heat Treatment	After Heat Treatment	Activity After Heat Treatment (%)
I, C225 (control I)[a]	6.18	2.37	38.4
II, GM5834 (control II)[a]	3.47	1.29	37.2
III, P05 (patient I)[b]	8.01	1.04	13.0
IV, P08 (patient I)[b]	7.12	.89	12.5
V, C225 + P05[c]	5.89	1.42	24.1

[a] Derived from normal subjects.
[b] Obtained from the lymphocytes of patient I.
[c] Contained half the volume of each of the enzyme extracts used in experiments I and III.

Table 4

MTHF Reductase Activity Before and After Heat Treatment in Crude and Partially Purified Lymphoblast Extracts

	Enzyme Activity (nmol HCHO/mg protein/h)					
	Crude Extracts			DEAE Adsorbed Fraction		
Strains	Before	After	% After	Before	After	% After
C225[a]	5.94	2.39	40.2	14.98	5.81	38.8
P05[b]	7.77	1.34	17.2	17.03	3.05	17.9

[a] Derived from a control subject.
[b] Derived from patient I.

lar to atherosclerosis (Kanwar et al. 1976). The severity of the clinical manifestations and the extent of the biochemical derangement seem to parallel the degree of enzyme deficiency (Erbe 1979, 1986; Rowe 1983). The onset of clinical symptoms varies, occurring from the neonatal period to adolescence. All previously reported patients had less than 20% of MTHF reductase activity in the absence of FAD. Large doses of oral folic acid produced a rapid decrease in homocystine excretion in some patients. However, their effect on the neurologic abnormalities was variable (Erbe 1979, 1986; Rowe 1983).

In contrast, patient I had no neurologic problems until the age of 42 years, when he had a mild stroke. However, he had multiple attacks of myocardial infarction between 35 and 45 years of age. Patient II did not have any history of neurological or vascular disease.

Decreased lymphocyte MTHF reductase activity (from 35% to 40% of the normal mean) was observed in lymphocyte extracts obtained from both patients I and II. However, we were unable to demonstrate a consistent decrease of MTHF reductase activity in cultured skin fibroblasts or lymphoblasts from the patients. It is well known that enzyme activities in cultured cells are highly variable. For example, acid β-glucosidase (N-acyl-sphingosyl-O-β-glycoside: glycohydrolase) activity in the supernatant derived from centrifugation of homogenate or sonicate of cultured cells was highly variable, leading to difficulties in distinguishing mutant cells from normal cells by using specific enzyme activity alone (Fabbro et al. 1987). In fibroblast cultures, cell density and growth rate had a pronounced effect on MTHF reductase activity (Rosenblatt and Erbe 1977*a*). In addition, enzyme activities were variable, depending on the growth phase (Rosenblatt and Erbe 1977*a*). Hence, our observations on MTHF reductase activities in cultured cells from the controls and from the patients were not unexpected; they demonstrated the need, when cultured cells are studied, for biochemical characterization of the enzyme to define a mutant.

Heat inactivation studies provided strong evidence to support the view that the MTHF reductase in our two patients was a mutant protein. Specific enzyme activity after heat treatment showed a distinct difference between the patients and the controls regardless of the source of enzyme preparation. Although both patients had subnormal or low normal serum cyanocobalamin, normal uptake and distribution of cyanocobalamin as well as normal methionine synthase activity in these patients excluded the possibility of abnormal cobalamin metabolism.

Genetic heterogeneity of severe MTHF reductase deficiency has been demonstrated elsewhere by thermostability studies of the enzyme (Rosenblatt and Erbe 1977*b*). However, the pattern of heat inactivation appeared to be different from that in our patients. Furthermore, the clinical and laboratory findings in our patients were entirely different from those of the previously reported cases. It had been known that MTHF reductase activity in some heterozygotes is only 30%–40% of the normal mean value (Wong et al. 1977*a*, 1977*b*). However, our study showed that the thermostability of the enzyme in these heterozygotes was essentially the same as that in the controls, providing further evidence for the existence of a hitherto undescribed mutant in our patients. Among intermediate homocysteinemics from our previous studies (Kang et al. 1986, 1987), only these two subjects were available for enzyme studies. Both had partial deficiency of lymphocyte MTHF reductase with decreased thermostability, suggesting that such variant(s) might not be uncommon among subjects with vascular diseases. Our preliminary report showed that 19 (23.2%) of 82 patients with coronary artery disease and two (5.1%) of 39 controls had thermolabile MTHF reductase (Kang et al. 1988).

In summary, the moderate deficiency of lymphocyte MTHF reductase in our two patients was associated with unique clinical and biochemical features. Hence, reliable methods for the detection and confirmation of mutant MTHF reductase may be important in the evaluation and treatment of these subjects.

Acknowledgment

The authors would like to thank Dr. H. C. Kwan of Northwestern University Hospital for the fibrinolytic assays and Dr. David S. Rosenblatt, Montreal Children's Hospital, for assaying the uptake and distribution of cyanocobalamin in cultured fibroblasts. This work was supported by research grant HL36135 from the National Heart, Lung and Blood Institute and by a Christ Hospital research grant.

References

Cooper, B. A., and D. S. Rosenblatt. 1987. Inherited defects of vitamin B12 metabolism. Pp. 291–320 *in* R. E. Olson, E. Bentler, and H. P. Broquist, eds. Annual Review of Nutrition. Vol. **7**. Annual Reviews, Inc., Palo Alto.

Dunn, R. T., and L. E. Foster. 1973. Radioassay of serum folate. Clin. Chem. **19**:1101–1105.

Erbe, R. W. 1979. Genetic aspects of folate metabolism. Pp. 293–354 *in* H. Harris and K. Hirschorn, eds. Advances in human genetics. Plenum, New York.

———. 1986. Inborn error of folate metabolism. Pp. 413–465 *in* R. L. Blakeley and V. M. Whitehead, eds. Folate and pterine. Vol. **3**. John Wiley & Sons, New York.

Fabbro, D., R. J. Desnick, and G. A. Grabowski. 1987. Gaucher disease: genetic heterogeneity within and among the subtypes detected by immunoblotting. Am. J. Hum. Genet. **40**:15–31.

Herbert, V., C. W. Gottlieb, and K. S. Lou. 1966. Hemoglobin-coated charcoal assay for serum B12. Blood **28**:130–132.

Jondal, M., G. Holm, and H. Wigzell. 1972. Surface markers on human T and B lymphocytes. I. A large population of lymphocytes forming nonimmune rosettes. J. Exp. Med. **136**:207–215.

Kang, S.-S., P. W. K. Wong, and N. Becker. 1979. Protein-bound homocyst(e)ine in normal subjects and patients with homocystinuria. Pediatr. Res. **13**:1141–1143.

Kang, S.-S., P. W. K. Wong, and C. Kay. 1982. The effect of D-penicillamine on protein-bound homocyst(e)ine in homocystinurics. Pediatr. Res. **16**:370–372.

Kang, S.-S., P. W. K. Wong, H. Y. Cook, M. Norusis, and J. V. Messer. 1986. Protein-bound homocyst(e)ine: a possible risk factor for coronary artery disease. J. Clin. Invest. **77**:P1482–1486.

Kang, S.-S., P. W. K. Wong, and M. Norusis. 1987. Homocysteinemia due to folate deficiency. Metabolism **36**:458–462.

Kang, S.-S., P. W. K. Wong, J. Zhou, J. Sora, M. Lessick, N. Ruggie, and G. Grcevich. 1988. Thermolabile methylenetetrahydrofolate reductase in patients with coronary artery disease. Metabolism **37**:611–613.

Kanwar, Y. S., J. R. Manaligod, and P. W. K. Wong. 1976. Morphologic studies in a patient with homocystinuria due to 5,10-methylenetetrahydrofolate reductase deficiency. Pediatr. Res. **10**:598–603.

Kutzbuch, C., and E. L. R. Stockstad. 1971. Mammalian methylenetetrahydrofolate reductase: partial purification, properties and inhibition by S-adenosylmethionine. Biochim. Biophys. Acta **250**:459–477.

Lou, K., C. W. Gottlieb, L. R. Wasserman, and V. Herber. 1965. Measurement of serum vitamin B12 level using radioisotope dilution on coated charcoal. Blood **26**:202–214.

Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.

Mudd, S. H., and H. L. Levy. 1983. Disorders of transsulfuration. Pp. 522–559 *in* J. B. Stanbury, J. B. Wyngaarden, D. S. Fredrickson, J. L. Goldstein, and M. S. Brown, eds. The metabolic basis of inherited disease. McGraw-Hill, New York.

Mudd, S. H., B. W. Uhlendorf, J. M. Freeman, J. D. Finkelstein, and V. E. Shih. 1972. Homocystinuria associated with decreased methylenetetrahydrofolate reductase activity. Biochem. Biophys. Res. Commun. **46**:905–912.

Rosenberg, L. E. 1983. Disorders of propionate and methylmalonate metabolism. Pp. 474–497 *in* J. B. Stanbury, J. B. Wyngaarden, D. S. Fredricksen, J. L. Goldstein, and M. S. Brown, eds. The metabolic basis of inherited disease. McGraw-Hill, New York.

Rosenblatt, D. S. and R. W. Erbe. 1977*a*. Methylenetetrahydrofolate reductase in cultured human cells. I. Growth and metabolic studies. Pediatr. Res. **11**:1137–1141.

———. 1977*b*. Methylenetetrahydrofolate reductase in cultured human cells. II. Genetic and biochemical studies of methylenetetrahydrofolate reductase deficiency. Pediatr. Res. **11**:1141–1143.

Rowe, P. B. 1983. Inherited disorders of folate metabolism. Pp. 498–521 *in* J. B. Stanbury, J. B. Wyndgaarden, D. S. Fredrickson, J. L. Goldstein, and M. S. Brown, eds. The metabolic basis of inherited disease. McGraw-Hill, New York.

Rudzki, Z., M. Nazaruk, and R. J. Kimber. 1976. The clinical value of the radioassay of serum folate. J. Lab. Clin. Med. **87**:859–867.

Sardharwalla, I. B., B. Fowler, A. J. Robins, and G. M.

Komorower. 1974. Detection of heterozygotes for homocystinuria: study of suphur containing amino acids in plasma and urine after L-methionine loading. Arch. Dis. Child. **49**:553–559.

Smolin, L. A., and N. G. H. Benevenga. 1982. Accumulation of homocyst(e)ine in vitamin B-6 deficiency: a model for the study of cystathionine-B-synthase deficiency. Nutrition **112**:1264–1272.

Wong, P. W. K., P. Justice, and S. Berlow. 1977*a*. The detection of homozygotes and heterozygotes with methylenetetrahydrofolate reductase deficiency. J. Lab. Clin. Med. **90**:283–287.

Wong, P. W. K., P. Justice, M. Hruby, E. B. Weiss, and E. Diamond. 1977*b*. Folic acid non-responsive homocystinuria due to methylenetetrahydrofolate reductase deficiency. Pediatrics **59**:749–754.